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(54) Title: 3'-END BLOCKED OLIGONUCLEOTIDES

(57) Abstract

The invention provides oligonucleotides having 3'-ends blocked by cap structures and having one or more artificial internucleoside linkage. Such oligonucleotides are resistant to *in vivo* degradation and extension, and thus have superior half-life *in vivo*.

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3'-END BLOCKED OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to antisense oligonucleotide therapy. More particularly, the invention relates to the production of oligonucleotides suitable for *in vivo* therapeutic use, and to the use of such oligonucleotides in therapeutic treatment of human disease.

Summary of the Related Art

The use of an antisense oligonucleotide approach for the treatment of human disease is a promising development in the fields of medicine related to antiviral therapy and therapy for genetic disorders.

In the last several years, it has been demonstrated that oligonucleotides are capable of inhibiting the replication of certain viruses in tissue culture systems.

Zamecnik and Stephenson, Proc. Natl. Acad. Sci. U.S.A. 75: 280-284 (1978), first showed oligonucleotide-mediated inhibition of virus replication in tissue culture, using Rous Sarcoma Virus.

Zamecnik et al., Proc. Natl. Acad. Sci. U.S.A. 83: 4143-4146 (1986), demonstrates inhibition in tissue culture of the HTLV-III virus (now called HIV-1) associated with AIDS.

More recently, it has been shown that modified oligonucleotides, having altered internucleoside linkages, provide greater efficacy in virus inhibition in *in vitro* tissue culture systems.

Agrawal et al., Proc. Natl. Acad. Sci. U.S.A. 85: 7079-7083 (1988), teaches inhibition in tissue culture of HIV-I with increased efficacy, using oligonucleoside phosphoramidates and phosphorothioates.

Sarin et al., Proc. Natl. Acad. Sci. U.S.A. 85: 7448-7451 (1988), teaches inhibition in tissue culture of HIV-1 with increased efficacy, using oligonucleoside methylphosphonates.

Agrawal et al., Proc. Natl. Acad. Sci. U.S.A. 86: 7790-7794 (1989) teaches nucleotide sequence specific inhibition of HIV-1 in both early-infected and chronically infected cell cultures, using oligonucleotide phosphorothioates.

Leiter et al., Proc. Natl. Acad. Sci. U.S.A. 87: 3430-3434 (1990), teaches inhibition in tissue culture of influenza virus replication by oligonucleotide phosphorothioates.

In addition, oligonucleotides have been used to modulate normal cellular processes, suggesting a potential use in the treatment of genetic disorders.

Goodchild et al., Arch. Biochem. Biophys. 264: 401-409 (1988), teaches inhibition of rabbit β-globin synthesis by oligonucleotides in a cell-free system.

Temsamani et al., Journal of Biological Chemistry (USA) 266: 468-472 (1991), teaches inhibition of spliceosome assembly by oligonucleoside methylphosphonates.

The inhibition of viruses and modulation of normal cellular processes indicates some promise for the use of an antisense oligonucleotide approach for the treatment of viral disease and genetic disorders. However, antisense oligonucleotide therapy depends upon the *in vivo* specificity and efficacy of oligonucleotides, both of which are related to oligonucleotide length, base composition and hybridization properties. Thus, if oligonucleotides are rapidly degraded *in vivo* to produce shorter degradation products, decreased efficacy may result, and the loss of specificity could lead to toxic side effects. Consequently, concerns arise about developing oligonucleotides that are resistant to degradation *in vivo*. Oligonucleotides having modified internucleoside linkages have been used by the previously-cited investigators toward this end.

Agrawal and Sarin, Advanced Drug Delivery Reviews, Elsevier Press, in press (1990), teaches that unmodified oligonucleotides are poor inhibitors of virus replication relative to modified (resistant) oligonucleotides.

Shaw et al., Nucleic Acids Res. 19: 747-750 (1991), teaches that otherwise unmodified oligonucleotides become more resistant to nucleases *in vitro* when they are blocked at the 3' end by certain capping structures and that uncapped oligonucleoside phosphorothioates also are not degraded *in vitro*.

Unfortunately, virtually nothing is known about the stability or biodistribution of modified or unmodified oligonucleotides *in vivo*, which is particularly relevant if oligonucleotides are to be used for human therapy. Since *in vitro* models cannot be made to predict the stability and bioavailability of oligonucleotides *in vivo*, there is a need for systems that can directly provide such information. Moreover, if such *in vivo* data shows that existing oligonucleotides are not sufficiently stable, there will be a need for oligonucleotides that can resist the degradative influences of intrinsic

enzymatic activities *in vivo*. Ideally, structural motifs associated with increased stability *in vivo* should be identified, and *in vivo* systems should be developed which allow simple and convenient comparisons for the optimization of *in vivo* stability.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structure of oligonucleotides used in Examples 1-7.

Figure 2. Examples of 3'-hydroxyl cap structures useful in forming the oligonucleotides of the present invention. R = an organic group, e.g., alkyl, aryl, cyclic, cholesteryl, etc.; X = O, S, Se or NHR; Y = O, S, Se or NHR; Z = O, S, or NH; and B = purine or pyrimidine base.

Figure 3. Comparative stability of uncapped, 5'-capped, 3'-capped and 3',5'-capped oligonucleoside phosphorothioate in monkey serum. Oligonucleotides were incubated in monkey serum at 37°C and at time points (shown above the lanes in hour), aliquots were withdrawn, extracted and analyzed on gel electrophoresis. In lane 6 hr. from 5'-capped, most of the incubation mixture was lost during handling.

Figure 4. Stability of oligonucleoside phosphorothioate in urine, collected from the mice receiving 30 mg/kg of oligonucleotide intravenously. The urine was collected up to 24 hour post-dosing, extracted and analyzed by gel electrophoresis. The lanes under control are oligonucleotides before administration and lanes under urine (0-24 hours) are oligonucleotides recovered from urine. Diffused band of 3'-capped oligonucleotide is due to the low specific activity (Table 1).

Figure 5. Status of the oligonucleoside phosphorothioate in mice kidney after 24 hour post-dosing intravenously. The lanes shown control are oligonucleoside before administration and lanes under kidney (24 hours) are the oligonucleoside extracted from kidney.

Figure 6. Gel electrophoresis of oligonucleoside phosphorothioate extracted from the liver after 24 hours post-dosing in mice, intravenously. The lanes under control are oligonucleoside phosphorothioate before administration and lanes under liver (24 hours) are oligonucleoside phosphorothioate extracted from liver.

BRIEF SUMMARY OF THE INVENTION

The invention relates to oligonucleotides that are useful in antisense oligonucleotide-based therapeutic approaches. More particularly the invention relates to oligonucleotides that have sufficient specificity and efficacy *in vivo* to be useful in therapeutic treatment of human disease. The invention provides oligonucleotides that possess greater *in vivo* specificity and efficacy than oligonucleotides known in the art. The greater specificity and efficacy of oligonucleotides according to the invention arises from their inherent resistance to exonucleolytic digestion by intrinsic nucleases. This resistance is the product of two structural features of oligonucleotides according to the invention: (1) the presence of one or more artificial internucleoside linkage and (2) the presence of a particular cap structure at the 3' end of the molecule.

For the first time, the invention provides *in vivo* pharmacokinetic information about modified and unmodified oligonucleotides. This information cannot be extrapolated from *in vitro* results, and indeed surprising differences are observed between *in vivo* and *in vitro* results. The invention provides such information through the use of a convenient method for assessing whether any given oligonucleotide has the resistance to nucleolytic degradation necessary to provide it with the specificity and efficacy required to use as an antisense oligonucleotide in a therapeutic approach to the treatment of human disease. This method uses a mouse model to assess resistance of a radiolabelled oligonucleotide to *in vivo* nucleases by examining the status of oligonucleotides present in urine and homogenized tissues of organs. The method also allows assessment of bioavailability of oligonucleotides by measuring the oligonucleotide content of various organs. By using the method of the invention, one skilled in the art can readily determine whether any oligonucleotide having the structural features of oligonucleotides according to the invention also is resistant to nucleolytic degradation *in vivo*. This method allows the skilled artisan to make such assessments by protocol, without undue experimentation.

DETAILED DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In a first aspect the invention provides oligonucleotides that are resistant *in vivo* to both degradation and extension by intrinsic enzymatic activities. These oligonucleotides according to the invention have greater specificity and increased half-life, relative to existing oligonucleotides. Such oligonucleotides are well-suited for use in the treatment of virus infections or disorders of gene expression. Virus infections include infections by DNA, RNA, and retroviruses. Disorders of gene expression include inherited genetic defects and disorders resulting from abnormal gene expression, or from expression of abnormal genes, e.g., oncogene expression associated with neoplasia.

The therapeutic approach using antisense oligonucleotides is based on the principle that an appropriate length of oligonucleotide complementary to the target can disrupt the function of the target, which could be a viral or cellular gene. The specificity of antisense oligonucleotides results from the formation of Watson-Crick base pairing between the heterocyclic bases on the oligonucleotide and complementary bases on the target nucleic acid. A nucleotide sequence of 16 nucleotides in length will be expected to occur randomly at about every 4^{16} or $4x10^9$ nucleotides. Thus such a sequence might be expected to occur only once in the human genome. In contrast, a nucleotide sequence of 10 nucleotides in length would occur randomly at about every 4^{10} or $1x10^6$ nucleotides. Thus such a sequence might be present thousands of times in the human genome. Consequently, oligonucleotides of greater length are more specific than oligonucleotides of lesser length and are less likely to lead to any toxic complications that might result from nonspecific hybridization. In addition, longer oligonucleotides show greater inhibitory effects upon HIV in tissue culture, within certain limits (i.e., 25-mer > 20-mer > 15-mer > 10-mer). Thus oligonucleotide length should exceed certain limits for purposes of specificity and effectiveness.

In vivo degradation of oligonucleotides produces oligonucleotide breakdown products of reduced length. Such breakdown products are more likely to engage in non-specific hybridization and are less likely to be effective, relative to their full-length counterparts. Thus, it is desirable to produce oligonucleotides that are resistant to degradation in the body. Preferably, such oligonucleotides should be bioavailable to the various organs and tissues of the body as well. Oligonucleotides according to the invention resist degradation by intrinsic nucleolytic activities and are bioavailable to

many organs and tissues. Thus in this aspect, the invention provides oligonucleotides that are well suited to act effectively and specifically in antisense oligonucleotide therapeutic approaches. In addition, *in vivo* metabolism of oligonucleotides results in extension of oligonucleotides in certain tissues, including at least liver, kidney, small intestine and large intestine. This can lead to reduced bioavailability of particular oligonucleotides and can lead to reduced specificity and to potentially mutagenic side effects.

Oligonucleotides according to the invention are resistant to both *in vivo* degradation and extension due to two structural features. The first feature is the presence of one or more internal artificial internucleoside linkages. Examples of such linkages that may be substituted for phosphodiester linkages include phosphorothioates, methylphosphonates, sulfone, sulfate, ketyl, phosphorodithioates, various phosphoramidates, phosphate esters, bridged phosphorothioates and bridged phosphoramidates. Such examples are illustrative, rather than limiting, since other internucleoside linkages are known in the art. See, e.g., Cohen, Trends in Biotechnology, (1990). The synthesis of oligonucleotides having one or more of these substituted for phosphodiester internucleoside linkages is well known in the art, which includes synthetic pathways for the production of oligonucleotides having mixed internucleoside linkages. The second feature of oligonucleotides according to the invention is the presence of a cap structure at the 3'-OH of the molecule. This cap blocks access to the 3' hydroxyl functional group, thus rendering the molecule more resistant to both extension 3' exonucleolytic activity, which is the primary intrinsic mediator of *in vivo* oligonucleotide degradation. Cap structures according to the invention include N-Fmoc-O'-DMTr-3-amino-1,2-propanediol, as well as the structures shown in Figure 2. Such examples are merely illustrative, however, since many blocking groups are known in the art and those skilled in the art will recognize how to attach such groups to the 3' end of the oligonucleotide. Thus, for purposes of the invention, a cap structure is construed to encompass any blocking group that restricts access to the 3' hydroxyl of an oligonucleotide, thereby rendering the oligonucleotide resistant to *in vivo* degradation or extension. For purposes of the invention, an oligonucleotide is considered to be rendered resistant to *in vivo* degradation if its *in vivo* half life is longer than that of an oligonucleotide having all phosphodiester internucleoside linkages, but otherwise being of

identical length and sequence. Preferably, the resistant oligonucleotide will have an *in vivo* half life that is longer than that of an uncapped oligonucleoside phosphorothioate of identical length and sequence.

In a second aspect, the invention provides a convenient method for assessing whether any particular oligonucleotide constitutes an oligonucleotide according to the invention. More particularly, the invention provides a convenient method for determining whether an oligonucleotide having one or more internal internucleoside linkage that is not a phosphodiester linkage, as well as having a 3' cap structure, is resistant to *in vivo* degradation. The method of the invention also allows convenient assessment of bioavailability of such oligonucleotides, which is preferable in certain embodiments of the oligonucleotides of the invention. Thus the invention provides a convenient method for assessing, without undue experimentation, whether a particular oligonucleotide possesses characteristics that make it desirable for use in therapeutic approaches involving antisense oligonucleotides.

In this aspect, the invention utilizes methods known in the art for synthesis of radioactively labelled oligonucleotides having one or more internal internucleoside linkage that is not a phosphodiester linkage, as well as having a cap structure attached to the 3' end. Such oligonucleotides are administered to mice in a physiologically acceptable carrier by either intravenous or intraperitoneal injection. After an appropriate interval, urine is collected from the treated mouse and the status of the oligonucleotides present therein is determined by PAGE and autoradiography. Bioavailability is determined by homogenization of organs and measurement of radioactivity therein. Finally, status of oligonucleotides in various organs is determined by extraction of the oligonucleotides from the homogenized organ tissues, followed by analysis using PAGE and autoradiography.

The invention further provides an even simpler assay which provides some preliminary information about oligonucleotide stability. This assay involves incubation of the oligonucleotides in the presence of monkey serum, followed by extraction of the oligonucleotides and analysis of degradation using PAGE and autoradiography.

The following examples are provided to further illustrate aspects of the invention and are not limiting in nature.

Example 1

Synthesis of Oligonucleoside Phosphorothioates

Oligonucleoside phosphorothioates were synthesized on a Model 8700 automated synthesizer (Milligen-Biosearch, Burlington, MA) using H-phosphonate chemistry on controlled pore glass (CPG), followed by oxidation with 0.2M sulfur in carbon disulfide/pyridine/triethylamine (9:9:1, v/v). Synthesis was carried out on a 5x10 micromolar scale. Oligonucleoside phosphorothioates were purified by low pressure ion exchange chromatography (DEAE-cellulose, DE-50 Whatman), followed by reverse phase chromatography (C_{18}) and dialysis. A detailed description of the H-phosphonate approach to synthesizing oligonucleoside phosphorothioates is given in Agrawal and Tang, Tetrahedron Letters 31: 7541-7544 (1990). In addition, synthesis of oligonucleoside methylphosphonates, phosphorodithioates, phosphoramidates, phosphate esters, bridged phosphoramidates and bridged phosphorothioates is known in the art. See e.g., Agrawal and Goodchild, Tetrahedron Letters 28: 3539 (1987); Nielsen et al., Tetrahedron Letters 29: 2911 (1988); Jager et al., Biochemistry 27: 7237 (1988); Uznanski et al., Tetrahedron Letters 28: 3401 (1987); Bannwarth, Helv. Chim. Acta 71: 1517 (1988); Crosstick and Vyle, Tetrahedron Letters 30: 4693 (1989); Agrawal et al., Proc. Natl. Acad. Sci. USA 87: 1401-1405 (1990).

Example 2

Synthesis of Capped Oligonucleoside Phosphorothioates

5'-capped oligonucleoside phosphorothioates were prepared by carrying out the last coupling, after the assembly of the required sequence, with N-Fmoc-O'-DMTr-3-amino-1,2-propanediol-H-phosphonate. The 5'-capped oligonucleoside H-phosphonate was then oxidized with sulfur. 3'-capped oligonucleoside phosphorothioates were assembled on N-Fmoc-O'-DMTr-3-amino-1,2-propanediol-CPG, followed by sulfur oxidation. Combination of these procedures was used to produce 3',5'-capped oligonucleoside phosphorothioates. See Figure 1.

Alternatively, oligonucleoside phosphorothioates having other 3' or 5' cap structures, (see, e.g., Figure 2), are prepared by substituting the phosphonate or CPG-derivatized cap structures for the N-Fmoc-O'-DMTr-3-amino-1,2-propanediol-H phosphonate or CPG in the capping procedure. Similarly, capped, modified oligonucleotides other than oligonucleoside phosphorothioates are prepared in an analogous manner by appending the capping procedure to the appropriate synthetic procedure.

Example 3

Preparation of ^{35}S-labelled Oligonucleoside Phosphorothioate

Five milligrams of CPG-bound oligonucleoside H-phosphonate was oxidized with a mixture of $^{35}S_8$ (5mCi, 1Ci/mg, Amersham, Arlington Heights, Illinois) in 40 microlitres carbon disulfide/pyridine/triethylamine (9:9:1). After 30 minutes, 100 microlitres cold S_8 in the same solvent mixture was added and the reaction was allowed to continue for 60 minutes. The solution was removed and the support was washed three times with 500 microlitres carbon disulfide and three times with 700 microlitres acetonitrile. The product was deprotected in concentrated ammonia at 55°C for 14 hours, evaporated, and desalted using Sep paK^{TM} C_{18} column (Waters, Milford, MA). The resultant product was purified by PAGE (20% polyacrylamide, 7M urea). The appropriate band was cut under UV shadowing, extracted from the gel and desalted. Yield was five A_{260} units or 150 micrograms. Specific activity was $5x10^9$ cpm/micromole or 440 nanocuries/microgram.

Other modified oligonucleotides can be labelled according to standard procedures, using ^3H or ^{14}C as label.

Example 4

Assessment of Oligonucleotide Stability in Monkey Plasma

Eighty micrograms at ^{35}S-labelled oligonucleoside phosphorothioate (capped or uncapped, specific activity 1.3 mCi/mg) was incubated with 50 microlitres monkey serum at 37°C. Aliquots were removed at time points and treated with proteinase K, (2 mg/ml, final concentration) in 0.5% SDS, 10mM NaCl, 20mM Tris•Cl (pH 7.6), 10mM EDTA for one hour at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. Recovered oligonucleotides were then analyzed by PAGE (20% polyacrylamide/7M urea) followed by autoradiography.

Results are shown in Figure 3. Uncapped and 5'-capped oligonucleoside phosphorothioates were degraded extensively within 24 hours. In contrast, 3'-capped and 3',5'-capped oligonucleoside phosphorothioates were stable after 24 hours. This indicates that degradation in monkey serum is primarily due to 3' exonucleases. Ladder formation indicates absence of significant levels of endonucleolytic activity.

When ^3H or ^{14}C-labelled oligonucleotides are used, autoradiography is carried out through the use of an appropriate enhancing fluorophore.

Example 5

In Vivo Oligonucleotide Stability Assessed by Urinary Analysis

Male CDC2F1 mice (average weight 20 grams) were treated by intravenous or intraperitoneal injection with a 30 mg/kg dose of oligonucleotides dissolved in 200 microlitres physiological saline. Each capped or uncapped oligonucleotide was administered to three mice. Urine was collected separately from each animal up to 24 hours post-dosing, then extracted with as in Example 4, and analyzed for radioactivity. Radioactivity was also measured from cage rinse to account for urine spill. Analysis was by PAGE (20% polyacrylamide, 7M urea) followed by autoradiography. The results are shown in Table 1 below.

Twenty-four hours after dosing, about 30% of oligonucleoside phosphorothioates were excreted, whether capped or uncapped. Excreted uncapped and 5'-capped oligonucleoside phosphorothioates were extensively degraded, as shown in Figure 4. Excreted 3'-capped and 3',5'-capped oligonucleoside phosphorothioates, in contrast, demonstrated virtually no degradation. This indicates that *in vivo* degradation of oligonucleoside phosphorothioates excreted in urine is mediated by 3'-exonuclease activity which can be inhibited by adding a cap to the 3' hydroxyl group of the oligonucleotide.

Table 1

URINARY EXCRETION OF OLIGONUCLEOTIDES IN MICE*

Intravenous Administration

Mouse Number	Oligo-nucleotide Number	Dose Administered (25 mg/kg;(Ci)	% of Dose Recovered Recovered in Urine		
			Urine	Cage Rinse	Total
1	uncapped	8.61	18.7	8.48	27.2
2	uncapped	8.61	26.4	8.55	35.0
3	uncapped	8.61	22.5	3.08	25.6
4	5'-capped	9.39	22.2	3.91	26.1
5	5'-capped	9.39	16.4	8.42	24.8
6	5'-capped	9.39	11.4	11.9	23.3
7	3'-capped	4.99	25.9	5.75	31.7
8	3'-capped	4.99	18.1	7.70	25.8
9	3'-capped	4.99	23.3	4.89	28.2
10	3',5'-capped	6.47	17.6	8.38	26.0
11	3',5'-capped	6.47	22.1	7.59	29.7
12	3',5'-capped	6.47	12.7	10.70	23.4

Example 6

Biodistribution of Oligonucleotides

Following the urinary oligonucleotide analysis of Example 5, the animals were sacrificed and autopsied, and all organs were removed. Each organ type was homogenized, lysed in buffer and assayed for radioactivity. Biodistribution of oligonucleotides is shown in Table 2 below. All types of capped and uncapped oligonucleoside phosphorothioates tested were bioavailable in most of the tissues of the organs 24 hours post-dosing. The concentration of oligonucleoside phosphorothioate in each tissue was independent of the presence, absence, or location of capping. Oligonucleotide concentration was highest in kidney, although total mass of oligonucleotide was highest in liver.

TABLE 2

TISSUE LEVELS OF OLIGONUCLEOTIDES IN MICE (24-HR POST-DOSING)*

(μg equivalents of oligonucleotide/gram of tissue)

Tissue	uncapped	5'-capped	3'-capped	3', 5'-capped
Kidney	195.00	230.00	184.67	200.33
Liver	27.33	43.60	34.93	37.70
Large Intestine	15.03	21.03	19.50	23.32
Small Intestine	10.68	14.20	12.87	12.91
Stomach	6.28	7.26	8.59	7.59
Spleen	6.03	12.40	9.41	13.13
Heart	4.16	7.02	5.23	6.31
Lung	3.81	5.41	4.33	5.90
Muscle	3.29	4.38	3.39	4.61
Plasma	2.85	1.98	3.16	1.70
Testes	1.64	2.14	1.56	1.73
Brain	0.27	0.26	0.34	0.23

*30 mg oligonucleotide/kg body weight, intravenously.

Example 7

Status of Oligonucleotides in Organs

Homogenized kidney or tissue from Example 6 was treated with proteinase K (2 mg/ml final concentration) in extraction buffer (0.5% SDS, 10 mM NaCl, 20mM Tris•HCl pH 7.6, 10mM EDTA) for two hours at 37°C. Samples were then extracted twice with phenol-chloroform and once with chloroform, followed by ethanol precipitation. Recovered oligonucleotides were fractionated by PAGE (20% polyacrylamide, 7M urea). The gel was then fixed in 10% acetic acid, 10% methanol and subjected to autoradiography. The results are shown for kidney in Figure 5. Uncapped oligonucleoside phosphorothioate extracted from kidney tissue was degraded to about 50%, and slower migrating bands (2-23 nucleotides in length) were also detected. Thus both degradation and extension of uncapped oligonucleoside phosphotothioates occurs in kidney. 5'-capped oligonucleoside phosphorothioates produced substantially identical results. In contrast, the great majority of bioavailable oligonucleotide was undegraded when either 3'-capped or 3',5'-capped oligonucleoside was used, and neither showed any sign of extension. Similar results were obtained for liver, as shown in Figure 6. No extension of 3' or 3',5'-capped oligonucleoside phosphorothioate was observed in small intestine either.

WHAT IS CLAIMED IS:

1. An oligonucleotide, wherein one or more internucleoside linkage is an artificial linkage, and wherein the oligonucleotide has a cap structure at a 3' hydroxyl; whereby the oligonucleotide is resistant to nucleolytic degradation *in vivo*.

2. An oligonucleotide according to claim 1, wherein one or more internucleoside linkage that is an artificial linkage is selected from the group consisting of phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate ester, bridged phosphoramidate, and bridged phosphorothioate.

3. An oligonucleotide according to claim 1, wherein the cap structure is selected from the group consisting of N-Fmoc-O'-DMTr-3-amino-1,2-propanediol, and the structures shown in Figure 2.

4. A therapeutic composition comprising an oligonucleotide of claim 1, in a physiologically acceptable carrier.

5. A therapeutic composition comprising an oligonucleotide of claim 2, in a physiologically acceptable carrier.

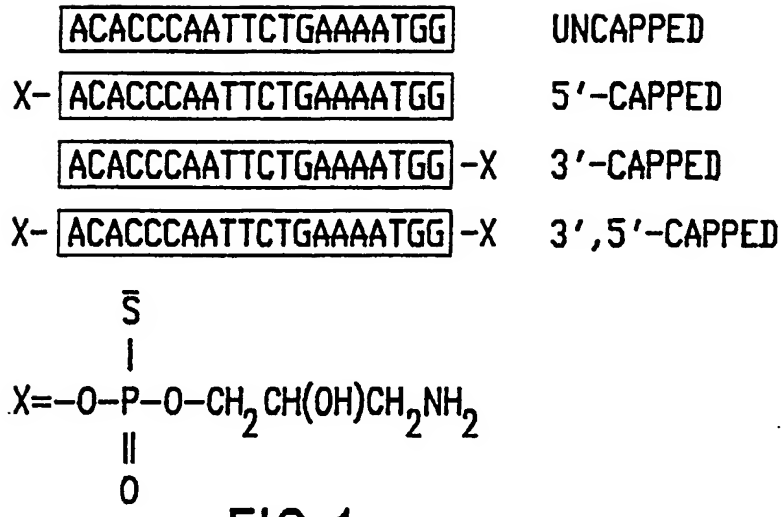
6. A therapeutic composition comprising an oligonucleotide of claim 3, in a physiologically acceptable carrier.

7. A method of treating a mammal infected with a virus, comprising administering a therapeutic composition according to claim 4.

8. A method of treating a mammal infected with a virus, comprising administering a therapeutic composition according to claim 5.

9. A method of treating a mammal infected with a virus, comprising administering a therapeutic composition according to claim 6.

10. A method of treating a mammal having a disorder of gene expression, comprising administering a therapeutic composition according to claim 4.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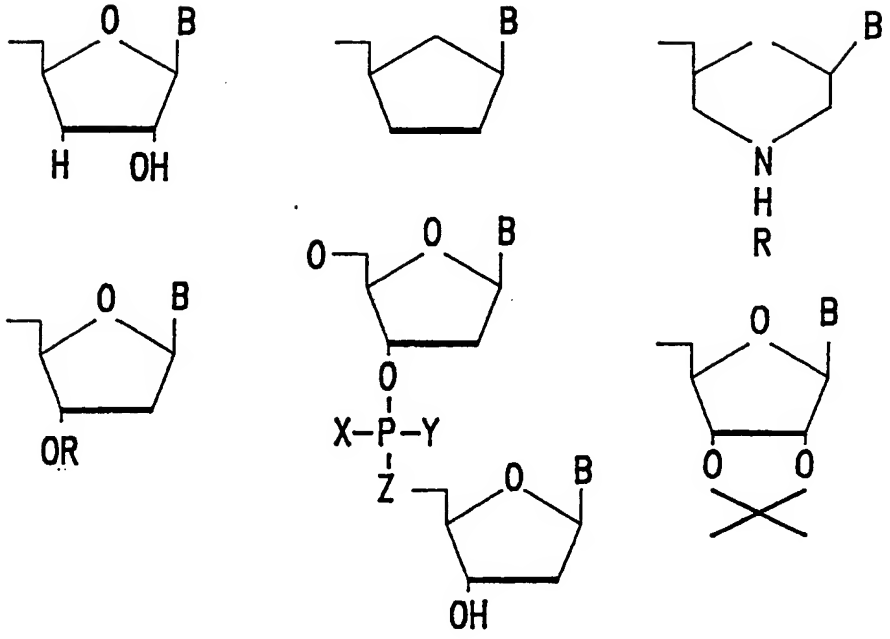
UNCAPPED
X-
ACACCCAATTCTGAAAATGG
5'-CAPPED
ACACCCAATTCTGAAAATGG
-X
3'-CAPPED
X-
ACACCCAATTCTGAAAATGG
-X
3',5'-CAPPED
S
X=-O-P-O-CH2 CH(OH)CH2NH2
O

FIG.1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B
H
OH
B
B
N
H
R
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OH
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B
O
O

FIG.2

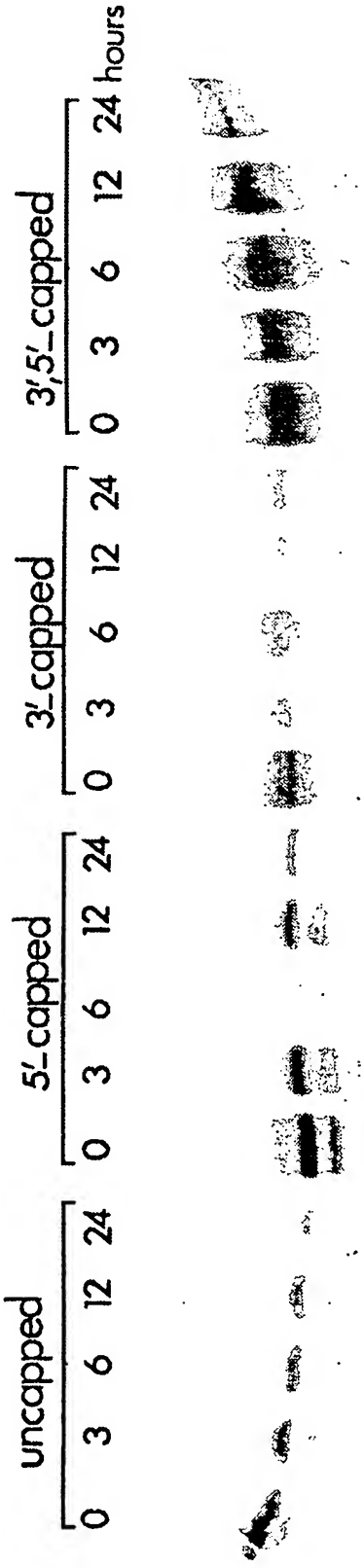
uncapped
0 3 6 12 24
5'_capped
0 3 6 12 24
3'_capped
0 3 6 12 24
3',5'_capped
0 3 6 12 24 hours

FIG. 3

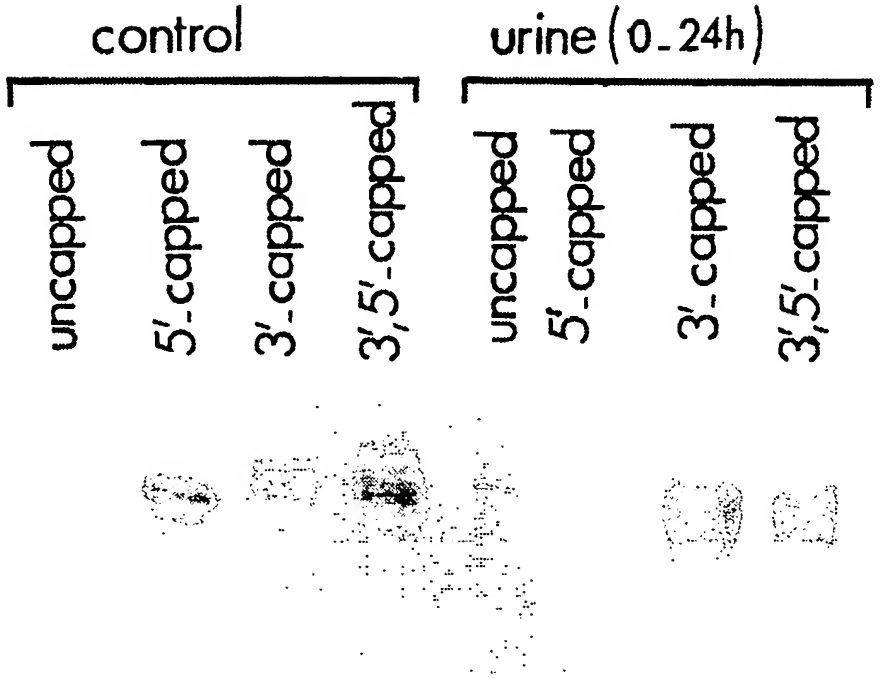
control
urine (0-24h)
uncapped
5'-capped
3'-capped
3',5'-capped
uncapped
5'-capped
3'-capped
3',5'-capped

FIG. 4

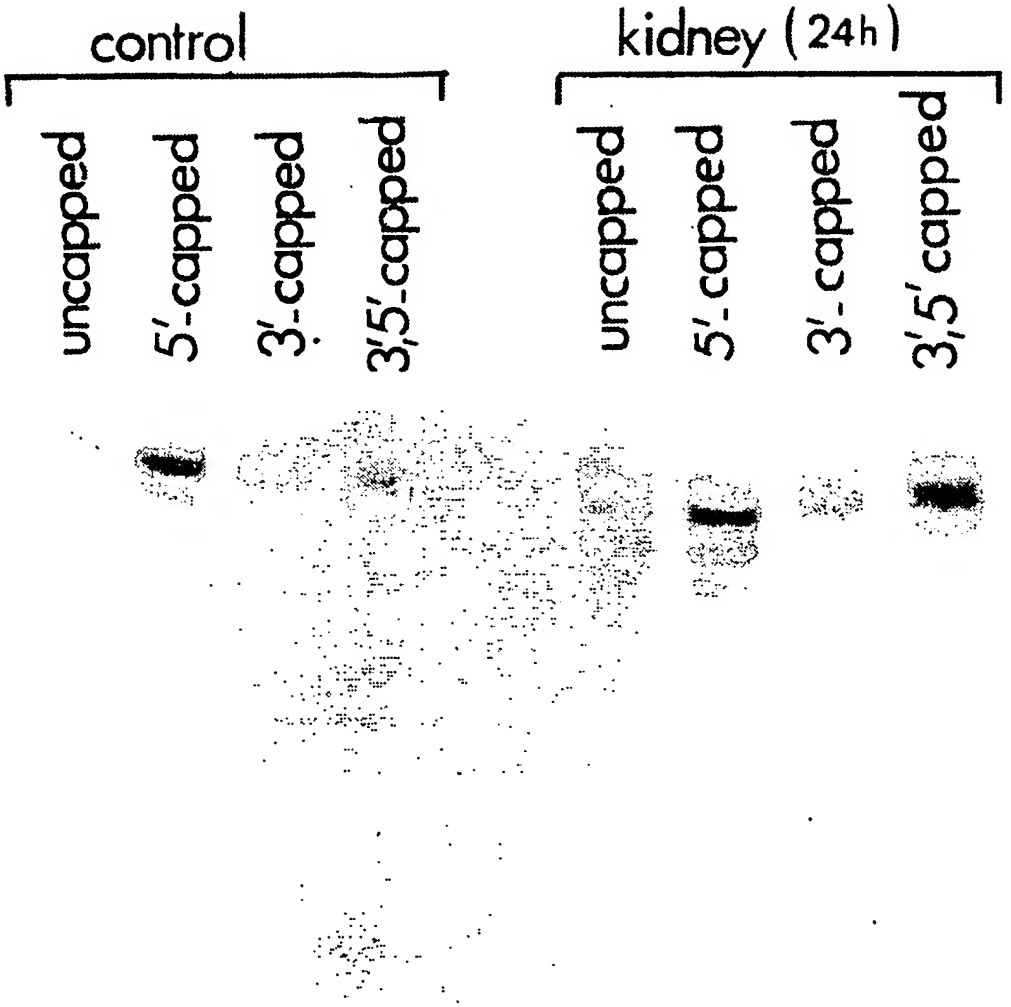
control
kidney (24h)
uncapped
5'-capped
3'-capped
3',5'-capped
uncapped
5'-capped
3'-capped
3',5'-capped

FIG. 5

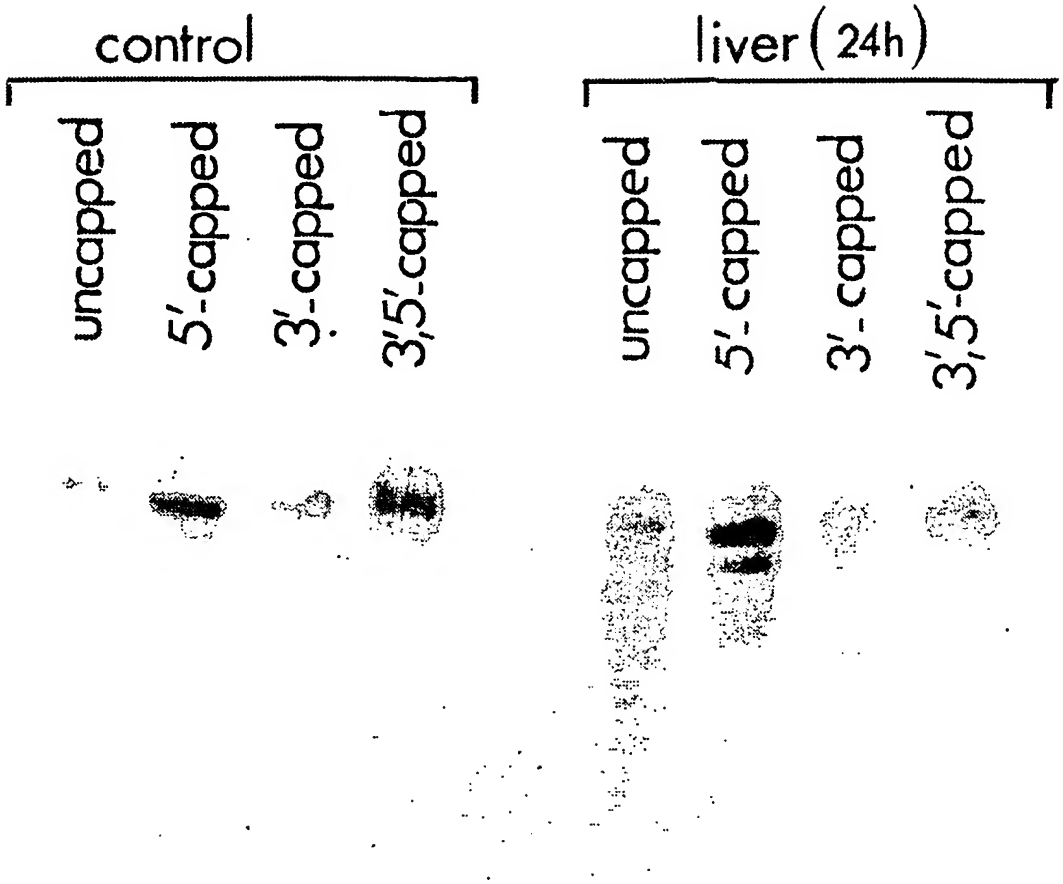
control
liver (24h)
uncapped
5'-capped
3'-capped
3',5'-capped
uncapped
5'-capped
3'-capped
3',5'-capped

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/03867

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07H21/00; A61K31/70

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07H ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	CHEMICAL ABSTRACTS, vol. 96, no. 19, 10 May 1982, Columbus, Ohio, US; abstract no. 156603S, S.D.PUTNEY ET AL.: 'A DNA Fragment with an alpha-Phosphorothioate Nucleotide at One End is Asymmetrically Blocked from Digestion by Exonuclease III and can be Replicated In Vivo.' page 201 ;column 1 ; see abstract	1-10
Y	& PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 78, no. 12, 1981, WASHINGTON US pages 7350 - 4; --- -/--	1-10

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 SEPTEMBER 1992	25.09.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SCOTT J.R. J.R.M. Scott

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 1, 5 January 1991, BALTIMORE US pages 468 - 472; J.TEMSAMANI ET AL.: 'Biotinylated Antisense Methylphosphonate Oligodeoxyoligonucleotides.' cited in the application see the whole document	1-10
Y	NUCLEIC ACIDS RESEARCH. vol. 19, no. 4, 25 February 1991, ARLINGTON, VIRGINIA US pages 747 - 750; J-P.SHAW ET AL.: 'Modified Deoxyoligonucleotides Stable to Exonuclease Degradation in Serum.' cited in the application see the whole document	1-10
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 83, no. 12, June 1986, WASHINGTON US pages 4143 - 6; P.C.ZAMECNIK ET AL.: 'Inhibition of Replication and Expression of Human T-Cell Lymphotropic Virus Type III in Cultured Cells by Exogeneous Synthetic Oligonucleotides Complementary to Viral RNA.' cited in the application see the whole document	1-10
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, no. 19, October 1988, WASHINGTON US pages 7079 - 83; S.AGRAWAL ET AL.: 'Oligodeoxynucleoside Phosphoramidates and Phosphorothioates as Inhibitors of Human Immunodeficiency Virus.' cited in the application see the whole document	1-10
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, no. 20, October 1989, WASHINGTON US pages 7790 - 4; S.AGRAWAL ET AL.: 'Inhibition of Human Immunodeficiency Virus in Early Infected and Chronically Infected Cells by Antisense Oligodeoxynucleotides and their Phosphorothioate Analogues.' cited in the application see the whole document	1-10

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, no. 20, October 1988, WASHINGTON US pages 7448 - 51; P.S.SARIN ET AL.: 'Inhibition of Acquired Immunodeficiency Syndrome Virus by Oligodeoxynucleoside Methylphosphonates.' see the whole document	1-10
Y	WO,A,8 807 542 (TECHNISCHE UNIVERSITAT EINDHOVEN) 6 October 1988 see claim 13	1-10
Y	EP,A,0 196 101 (CHIRON CORPORATION) 1 October 1986 see the whole document	1-10
Y	EP,A,0 203 870 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 3 December 1986 see abstract	1-10
Y	WO,A,8 905 358 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 15 June 1989 see the whole document	1-10
Y	WO,A,9 012 024 (WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY) 18 October 1990 see the whole document	1-10
Y	WO,A,9 012 022 (UNIVERSITY PATENTS INC.) 18 October 1990 see abstract; claims 1-4	1-10
P,X	NUCLEIC ACIDS RESEARCH. vol. 19, no. 20, 25 October 1991, ARLINGTON, VIRGINIA US pages 5743 - 8; G.D.HOKE ET AL.: 'Effects of Phosphorothioate Capping on Antisense Oligonucleotide Stability, Hybridization and Antiviral Efficacy Versus Herpes Simplex Virus Infection.' see the whole document	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03867

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7-10 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203867 SA 60562

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8807542	06-10-88	NL-A- 8700724	17-10-88
		EP-A- 0358657	21-03-90
		JP-T- 2503911	15-11-90
EP-A-0196101	01-10-86	CA-A- 1293938	07-01-92
		EP-A- 0398391	22-11-90
		JP-A- 62019096	27-01-87
EP-A-0203870	03-12-86	FR-A- 2582653	05-12-86
		JP-A- 62016500	24-01-87
WO-A-8905358	15-06-89	AU-A- 2782989	05-07-89
		EP-A- 0348458	03-01-90
		JP-T- 2502516	16-08-90
WO-A-9012024	18-10-90	CA-A- 2051692	07-10-90
WO-A-9012022	18-10-90	AU-A- 5418290	05-11-90